Cardiovascular Toxicology (2019) 19:548–564
https://doi.org/10.1007/s12012-019-09528-7

Intermittent Exposure to Chlorpyrifos Differentially Impacts Neuroreflex Control of Cardiorespiratory Function in Rats

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Published online: 16 May 2019

Abstract

Previous studies showed that chlorpyrifos (CPF) acute exposure impaired cardiorespiratory reflexes. Evidence also indicates that continuous exposure to organophosphorus compounds impairs cardiovascular function. However, the effect of intermittent exposure to CPF, as may be experienced in the real world, on tonic and reflex cardiorespiratory function remains unexplored. Wistar rats were injected with saline or CPF for 4 weeks (3 times/week) or 12 weeks (once/week) at the doses of 7 mg/kg and 10 mg/kg. After exposure, blood pressure (BP), heart rate (HR), respiratory rate (f_R), tidal volume (V_T), and minute volume (V_E) were recorded. Systolic BP and pulse interval (PI) variability, HR spectrum, spontaneous baroreflex and chemoreflex function were also evaluated. Plasma butyrylcholinesterase and brainstem acetylcholinesterase activities were quantified. Enzymatic activity of the CPF animals was reduced after both treatment periods. Baseline BP, HR, and f_R, as well as systolic BP and PI variability indices, did not change, after CPF treatment. V_T and V_E were elevated in CPF animals. CPF exposure increased the very low-frequency component of the HR spectrum. Baroreflex gain was reduced after CPF 4-week exposure. Chemoreflex bradycardia was reduced in the CPF-treated rats. These data show that intermittent exposure to CPF impairs cardiorespiratory function in rats. These results may have important clinical implications for workers seasonally exposed to these compounds.

Keywords Chlorpyrifos · Chemoreflex · Baroreflex · Cardiovascular variability · Butyrylcholinesterase · Acetylcholinesterase

Introduction

The health impact of acute and long-term exposure to pesticides has been a major concern, particularly in developing countries where consumption of these products has been rising for more than 25 years [1, 2], elevating the risk of exposure and poisoning. In particular, organophosphorus (OP) compounds are highly toxic pesticides widely involved in poisoning worldwide [3, 4].

Among the vulnerable populations are agricultural workers who directly handle pesticides, often without use of self-protection equipment, and this population is at high risk of exposure to doses that may result in acute and/or chronic poisoning [5–7]. Chronic poisoning, particularly in farmers, may vary according to the frequency of exposure, which is determined by the season, type of crop, and the periodicity of application recommended by the pesticide vendors [8–11]. It may also be associated with non-compliance with

Handling Editor: Kurt J. Varner.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12012-019-09528-7) contains supplementary material, which is available to authorized users.

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restricted re-entry guidelines imposed by regulatory agencies [12].

There is increasing evidence that either acute or repeated exposure to OP compounds may generate long-term neurological and neurobehavioral dysfunction [3, 6, 13–16]. These effects may or may not be associated with the well-known mechanism of toxicity of pesticides to inhibit esterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), leading to accumulation of acetylcholine within synaptic clefts [3, 13, 14].

There is evidence associating subchronic and chronic exposure to pesticides, particularly OPs, with cardiotoxicity (see Georgiadis et al. [17] for review). In this regard, most experimental studies have used continuous exposure protocols to evaluate long-term cardiotoxicity of OPs in animal models [18–25]. However, continuous exposure may not be applicable to a susceptible population, particularly farmers, where exposure to OPs is more likely to be intermittent. Moreover, the effect of long-term intermittent exposure to these compounds on neuroreflex regulation of cardiorespiratory function remains poorly explored.

Previous studies from our group, using a rat model of acute intoxication with CPF, a commonly used OP, showed an impairment of three homeostatic and protective reflexes involved in cardiorespiratory regulation: the baroreflex, the chemoreflex, and the Bezold–Jarisch reflex [26]. Impairment of cardiorespiratory reflexes, especially the chemoreflex and the baroreflex, is associated with the development and/or maintenance of long-term cardiorespiratory complications, such as systemic hypertension, heart failure, dyspnoea, and apnoea [27–33].

Given the dearth of knowledge regarding the effect of OP exposure on neuro-cardiorespiratory reflex function and the lack of experimental studies evaluating the effect of intermittent exposure, we studied baroreflex and chemoreflex function following intermittent CPF exposure in rats. We hypothesized that intermittent exposure would reduce baro- and chemoreflex responses as well as would affect respiratory function. Our study shows that intermittent exposure to CPF significantly disrupts both homeostatic and protective cardiorespiratory reflex function.

Materials and Methods

Animals

Adult male Wistar rats weighting between 200 and 300 g were used. The animals were provided by the animal facilities of the Health Sciences Center of the Federal University of Espírito Santo. Rats were housed in groups of 5/cage in a room with controlled temperature (20–24 °C) and humidity, with a 12-h light/dark cycle (lights on at 6:30 a.m.). Standard chow and tap water were available ad libitum.

Solutions

Chlorpyrifos solutions were prepared by dilution of a commercial formulation (CPF, Lorsban 480 BR®, 48% m/v, Dow Agrosciences Industrial Ltda) in saline (NaCl 0.9%). In order to achieve the specified doses applied for each group (*see below*), dilution was adjusted based on the content of the active ingredient specified in the formulation. Solutions were always freshly prepared and used on the same day. Control animals were treated with saline.

Experimental Protocol

Considering that exposure to pesticides in farmers may have different cycle lengths depending on the season, number of sprays per season, and type of crop [8–11, 34], we proposed a design of intermittent exposure at two time intervals, but administered with the same number of total doses per group. One group of animals was treated weekly with CPF or saline, for 12 weeks and another group of animals was treated three times a week, on alternating days, for 4 weeks. By adopting the same number of injections (total 12 administrations), within two time intervals, we could test whether longer or shorter intervals between exposures differentially impacts the cardiorespiratory function. The CPF doses chosen for treatment were 7 mg/kg and 10 mg/kg. The dose of 10 mg/kg corresponds to 1/3 of the dose that impaired cardiovascular function in a model of acute intoxication with CPF previously described by our group [26]. The 7 mg/kg corresponds to 2/3 of the 10 mg/kg dose. Either saline or CPF administration was performed through intraperitoneal injection to assure accurate and efficient delivery of doses. The same route of exposure to OP compounds has been used in other studies performed by our group [26, 35–37] as well as in studies from other groups [38, 39] and aims to reduce variability that would make evaluation of effects induced by the intoxication procedure difficult. CPF injections were always performed at the same time of day. According to the treatment (saline or CPF), dose (7 mg/kg and 10 mg/kg), and intervals of CPF treatment (4 weeks/3 times/week; 12 weeks/once/week), the final number of animals per groups is depicted in Table 1.

Animal weight and survival was monitored weekly from the beginning to the end of the treatment period. Three days after the treatment period ceased, animals were catheterized to allow pressure recordings (See *catheterization procedure* for details). This time interval between the last dose administration and begin of cardiorespiratory procedures was respected to avoid that effects assessed were associated with an acute exposure. At the end of the recordings, animals

Table 1 Number of animals for groups intermittently treated with saline (NaCl, 0.9%) or chlorpyrifos (CPF) at 7 mg/kg (CPF 7) and 10 mg/kg (CPF 10) for 4 weeks and 12 weeks

Groups	4 weeks/3 times/week ($N = 32$)	12 weeks/ once/week ($N = 39$)
Saline	9	13
CPF 7	14	14
CPF 10	9	12

were decapitated for collection of brainstem to measure AChE, and blood for measurement of BChE activity.

Catheterization Procedure and Pressure Recordings

Rats were anesthetized with tribromoethanol (250 mg/kg) and the femoral artery and vein were catheterized to allow pulsatile blood pressure recording and chemical administration, respectively. Rats were allowed to recover for 24 h between surgical procedures and cardiorespiratory reflex assessment. Blood pressure recordings were performed in awake rats using a pressure transducer connected to a computer recording system (Powerlab, ADInstruments, New Zealand). The mean (MBP), systolic (SBP) and diastolic blood pressure (DBP) were derived from pulsatile blood pressure (PBP) recordings using the systems software (LabChart®, AD Instruments, Bella Vista, NSW, Australia). Heart rate (HR) was derived from the interpulse interval also using the software.

Cardiovascular Variability

Recordings of PBP while animals rested for 10 min were used for analysis of spontaneous SBP and HR variability, using time and frequency domain analysis with CardioSeries software (version 2.3-http: http://www.danielpenteado.com), as described previously [40, 41].

Time Domain

For time domain analysis, variance, standard deviation (SD), and root mean square of successive interpulse interval differences (RMSSD) were calculated for systolic blood pressure (SBP) and pulse interval (PI). Data analysis was performed blindedly to treatment. If a recording presented a large number of artifacts, due to sudden movements of the animal or to interruptions in the recording signal, the animal was excluded from the analysis. For that reason, the number of animals used differed from the number of rats presented in some of the parameters analyzed.

Frequency Domain

For the frequency domain analysis, beat-to-beat series of PI recordings were converted into data points using an interpolation rate of 10 Hz and half overlap for sequential data sets containing 512 points by sequence. Non-stationary or aberrant segments containing spikes arising from animal movement or recording interruption were visually identified and excluded from the spectral analysis calculation. Thus, similar to time domain analysis, the number of animals differs according to the parameter analyzed. The spectrum was calculated using a Fast Fourier Transform (FFT) algorithm and was integrated into very low-frequency 0–0.2 Hz (VLF), low-frequency 0.2–0.75 Hz (LF), and high-frequency 0.75–0.3 Hz (HF) bands [41]. The results are presented in absolute and normalized power. When the analysis included normalized units, VLF was excluded and the LF/HF ratio was also calculated.

Spontaneous Baroreflex

Spontaneous baroreflex was evaluated using the time domain sequence, according to the method described by Fazan et al. [42] and reproduced by Simões et al. [41]. Baroreflex sequences were defined as blood pressure ramps, increasing or decreasing, of at least four arterial pulses, when the SBP changes were directly correlated with changes in PI and showed a linear correlation coefficient > 0.8. For the sensitivity or spontaneous baroreflex gain (sBRG), we considered the slope of the calculated linear regression for ascending and descending ramps. Finally, baroreflex effectiveness index (BEI) was calculated as the ratio between the number of baroreflex sequences and the total number of SBP ramps found.

Chemoreflex Activation

Chemoreflex activation was achieved by intravenous injections of randomly assigned doses of potassium cyanide (KCN; 10, 20, 40, 80 µg/rat; Impex, SP, Brazil) as described by Franchini and Krieger [43]. Peak hypertensive, bradycardic and tachypneic responses (see Respiratory recordings section for details) were taken after each dose of KCN. A minimum interval of 15 min between doses was waited or until baseline values had recovered.

Respiratory Recordings

Respiratory recordings were performed by the barometric technique [44] using a whole-body plethysmography chamber (volume: 6240 cm^3) for small animals, coupled to a ML141 Spirometer (PowerLab, ADInstruments, Australia). The chamber was tightly sealed before the KCN injections

and remained sealed for up to 2 min. Respiratory parameters recorded were respiratory frequency (f_R), tidal volume (V_T), and minute ventilation (V_E).

Measurement and Analysis of Respiratory Frequency (f_R)

Recordings of f_R were performed 20 s before and 20 s after KCN injections. The first 20 s was used as baseline frequency and 20 s after injections was used to measure the tachypneic response. Frequency was calculated manually for each 2-s interval and the number of cycles was multiplied by 30 to obtain the number of cycles per minute (cpm). The peak tachypneic response after each dose of KCN was considered for the analysis.

Measurement and Analysis of Tidal Volume (V_T) and Minute Ventilation (V_E)

Baseline values of V_T and V_E were derived from the recordings performed 20 s before KCN. These parameters could not be measured after KCN injections as this caused movement of the rat that interfered with the respiratory signal. The volume was calibrated by 3 repeated injections of 1 mL of air into the chamber using a syringe and the amplitude average in mV was calculated through the system software (LabChart software (ADInstruments, Australia). The average for each subinterval of 2 s in mV was also calculated, as previously described for the f_R, and values obtained were used to calculate V_T ($mL \cdot kg^{-1}$) and V_E ($mL \cdot kg^{-1} \cdot min^{-1}$) through the formula described by Drorbaugh and Fenn [45].

Enzymatic Assays

Blood and brainstem samples were collected following decapitation. Butyrylcholinesterase (BChE) activity in plasma and acetylcholinesterase (AChE) activity in the brainstem were measured. Decapitation avoided the use of anesthetics that interfere with AChE activity [46–50]. From all samples collected, some samples were randomly selected for measurements of enzymatic activity. Blood samples were collected in heparinized Eppendorf tubes and plasma obtained by centrifugation at 7200 G for 10 min at 4 °C (Universal 320R-Hettich; Tuttlingen, Germany). Samples were stored at − 80 °C until the enzymatic assay was performed.

Butyrylcholinesterase (BChE) Activity

BChE activity was measured in a semi-automated biochemical analyzer (Bio Plus®, BIO-2000 IL, Brazil) using a commercial kit (K094, Quibasa, MG, Brazil), according to the colorimetric method described by Dietz and colleagues [51], with modifications. The activity was expressed in International Units (U/L), where one U of cholinesterase is equivalent to the amount of enzyme that hydrolyzes one µmol of substrate/minute/mL of serum at 37 °C.

Acetylcholinesterase (AChE) Activity

Measurements of AChE were processed from brainstem samples. The enzyme activity was determined according to the method described by Ellman et al. [52] and modified by Lassiter et al. [53]. A homogenate of brainstem was produced using a Potter-Elverhjem homogenizer (Tecnal Equipamentos para Laboratórios Ltda, SP-Brazil) and processed in phosphate buffer (pH = 0.8, 0.1 M, Dinâmica Química Contemporânea Ltda, SP-Brazil) and Triton X-100 (1% v/v, Neon Comercial Ltda, SP-Brazil) in the proportion of 20 mg of tissue for 1 mL of buffer. A volume of 135 µl of the brainstem homogenate was added to a cuvette containing the following reagents: 35 µl of 0.01 M dithiobisnitrobenzoic acid (DTNB), 10 µL of 75 mM acetylthiocholine (ATCh), and 820 µL phosphate buffer, pH 8.0, to make a final volume of 1 mL. The development of color was analyzed at 412 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The AChE activity was expressed in millimoles of ATCh hydrolyzed per hour per milligram of protein. The protein content of brainstem homogenates was quantified by the method of Bradford [54], using bovine serum albumin as standard.

Data Analysis

A two-way ANOVA for repeated measures was used for analysis of hypertensive, bradycardic and tachypneic chemoreflex response data, as well as for body weight analysis. One-way ANOVA was used for all other parameters analyzed. Both ANOVA analyses were followed by the Bonferroni's *post hoc* test, when appropriate. Statistical details for one-way and two-way ANOVA are presented in Tables 1 and 2 of supplementary material. Data were analyzed with GraphPad Prism software version 5.00 for Windows (GraphPad Software®, San Diego California USA) and Statistical Package for the Social Sciences version 20.0 for Windows (SPSS Statistics, Chicago, IL, USA). Level of significance was set at $P < 0.05$ and data presented as mean ± standard error of the mean (SEM).

Results

Body Weight Gain and Survival

Body weight gain of animals is depicted in Fig. 1 and statistical details are presented at Online Resource 1. Animals treated for 4 weeks presented a significant weight gain over

Table 2 Baseline hemodynamic data and time domain systolic blood pressure (SBP) and pulse interval (PI) variability at the time domain

	4 weeks/3 times/week				12 weeks/once/week			
	SAL ($N = 9$)	CPF 7 ($N = 14$)	CPF 10 ($N = 9$)	F and P values	SAL ($N = 13$)	CPF 7 ($N = 14$)	CPF 10 ($N = 12$)	F and P values
DBP (mmHg)	95.67 ± 2.55	92.55 ± 2.79	93.38 ± 4.53	$F_{(2,29)} = 0.233$; $P = 0.793$	93.39 ± 2.32	90.22 ± 2.60	96.20 ± 2.76	$F_{(2,36)} = 1.358$; $P = 0.270$
SBP (mmHg)	129.36 ± 3.12	127.18 ± 2.55	122.81 ± 3.19	$F_{(2,29)} = 1.123$; $P = 0.339$	128.41 ± 2.85	127.51 ± 3.60	126.02 ± 4.08	$F_{(2,36)} = 0.112$; $P = 0.894$
MBP (mmHg)	111.00 ± 2.43	108.28 ± 2.59	102.69 ± 3.41	$F_{(2,29)} = 1.913$; $P = 0.166$	109.09 ± 2.34	107.03 ± 2.86	109.98 ± 2.99	$F_{(2,36)} = 0.310$; $P = 0.736$
HR (bpm)	357.81 ± 9.93	371.61 ± 9.45	343.10 ± 19.78	$F_{(2,29)} = 1.266$; $P = 0.297$	390.85 ± 16.02	409.14 ± 17.45	352.10 ± 16.45	$F_{(2,36)} = 2.961$; $P = 0.065$
	SAL ($N = 9$)	CPF 7 ($N = 13$)	CPF 10 ($N = 8$)	F and P values	SAL ($N = 12$)	CPF 7 ($N = 13$)	CPF 10 ($N = 10$)	F and P values
SBP variability								
SD (mmHg)	2.77 ± 0.21	3.40 ± 0.29	3.01 ± 0.31	$F_{(2,27)} = 1.325$; $P = 0.282$	2.98 ± 0.29	3.15 ± 0.36	3.02 ± 0.27	$F_{(2,32)} = 0.080$; $P = 0.923$
Variance ($mmHg^2$)	8.18 ± 1.14	12.84 ± 2.34	9.85 ± 1.85	$F_{(2,27)} = 1.446$; $P = 0.253$	9.83 ± 2.11	11.52 ± 3.13	9.81 ± 1.74	$F_{(2,32)} = 0.159$; $P = 0.854$
PI variability								
SD (ms)	3.20 ± 0.24	3.83 ± 0.33	4.18 ± 0.34	$F_{(2,27)} = 2.087$; $P = 0.144$	3.75 ± 0.25	3.88 ± 0.25	4.68 ± 0.47	$F_{(2,32)} = 2.293$; $P = 0.117$
Variance (ms^2)	10.84 ± 1.64	16.72 ± 2.54	18.57 ± 3.04	$F_{(2,27)} = 2.281$; $P = 0.122$	14.80 ± 1.98	15.96 ± 1.96	24.06 ± 4.70	$F_{(2,32)} = 2.803$; $P = 0.076$
RMSSD (ms)	3.73 ± 0.30	4.21 ± 0.35	4.69 ± 0.67	$F_{(2,27)} = 1.013$; $P = 0.377$	3.84 ± 0.46	4.18 ± 0.30	4.71 ± 0.38	$F_{(2,32)} = 1.231$; $P = 0.305$

Saline (SAL), chlorpyrifos 7 mg/kg (CPF 7), chlorpyrifos 10 mg/kg (CPF 10), diastolic blood pressure (DBP), mean blood pressure (MBP), heart rate (HR), systolic blood pressure (SBP), standard deviation (SD), pulse interval (PI), root mean square of the successive interval differences (RMSSD). One-way ANOVA

time ($P < 0.01$). However, no significant difference in weight for the treatment was observed among the different groups ($P > 0.05$), although statistical difference was observed for the interaction between treatment and time ($P < 0.05$, Online Resource 1). Animals treated for 12 weeks (Fig. 2b) also increased weight over time ($P < 0.01$), without significant differences among the treated groups ($P > 0.05$). Regarding impact on survival, CPF treatment with 10 mg/kg for 4 weeks, 3 times a week, leads to 14.6% mortality during the treatment period.

Butyrylcholinesterase (BChE) Activity

Animals treated with CPF for 4 weeks (Panel a), with either 7 mg/kg ($P < 0.05$) or 10 mg/kg, ($P < 0.05$) presented lower activities than the control group (Fig. 2a). The same effect was observed for both groups (CPF 7, $P < 0.01$; CPF 10, $P < 0.05$) of animals treated with CPF during 12 weeks (Fig. 2b). For statistical details see Online Resource 2.

Acetylcholinesterase (AChE) Activity

Animals treated for 4 weeks with CPF at 7 mg/kg ($P < 0.05$) and 10 mg/kg ($P < 0.01$) presented lower AChE activity within the brainstem when compared with control treated animals (Fig. 2c). A similar inhibition was observed by 12-week CPF treatment with the same doses (CPF 7, $P < 0.01$; CPF 10, $P < 0.01$, Fig. 2d; Online Resource 2).

Hemodynamic Data

Repeated exposure to CPF for 4 weeks did not affect baseline values of DBP, SBP, MBP, and HR and similar findings for baseline values resulted after 12-week treatment (Table 2).

Cardiovascular Variability

Parameters of SBP and PI variability of animals treated for 4 and 12 weeks are summarized in Table 2. No difference was observed in the SD and variance of SBP and PI as well as on RMSSD of different groups treated either during 4 or 12 weeks (Table 2).

HR Spectral Analysis

The very low-frequency band of HR variability from the CPF 10 group, treated for 4 weeks, was significantly increased when compared with saline ($P < 0.01$) and with the CPF 7 groups ($P < 0.01$) (Fig. 3a). In contrast, absolute power or normalized units of LF and HF did not differ

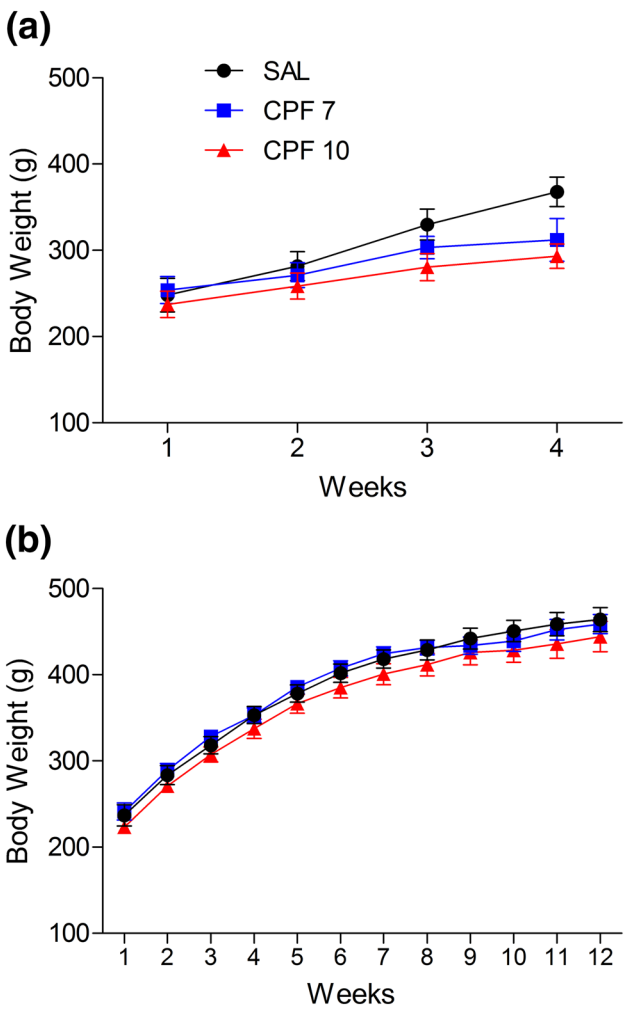

Fig. 1 Body weight gain of animals treated with saline (NaCl 0.9%, SAL—black circle) or chlorpyrifos (CPF) at 7 mg/kg (CPF 7—blue square) and 10 mg/kg (CPF 10—red triangle) during 4 weeks/3 times/week (Graph a, SAL, $N = 9$; CPF 7, $N = 14$; CPF 10, $N = 9$) and 12 weeks/once/week (Graph b, SAL, $N = 13$; CPF 7, $N = 14$; CPF 10, $N = 12$). Two-way ANOVA for repeated measures

among treated groups ($P > 0.05$, Fig. 3a, b), as well as no difference could be observed for LF/HF ratio among the groups studied (Fig. 3c).

Rats treated for 12 weeks with 10 mg/kg CPF exhibited a higher VLF band ($P < 0.01$) than the saline-treated group (Fig. 3d). Absolute power of LF and HF bands was not different among the groups studied ($P > 0.05$; Fig. 3d). Similarly to the 4-week treatment, neither LF and HF bands, in normalized units, nor the LF/HF ratio significantly differed among rats treated for 12 weeks (Fig. 3e, f). For statistical details see Online Resource 2.

Spontaneous Baroreflex

CPF treatment for 4 weeks, at both doses, decreased baroreflex gain of up ramps when compared with the saline group ($P < 0.05$; Fig. 4a). No significant difference was observed in baroreflex gain of down ramps among the groups treated for 4 weeks. As for all ramps, a significant reduction was only observed in CPF-treated animals at 7 mg/kg ($P < 0.05$) when compared with the saline control group. Only CPF 7 mg/kg treatment reduced BEI when compared with the saline group ($P = 0.005$; Fig. 4b, Online Resource 2). After 12 weeks of treatment, baroreflex gain as well as BEI did not differ among groups studied (Fig. 4c, d, Online Resource 2).

Chemoreflex Responses

Chemoreflex hypertensive and tachypneic responses among 4-week treated groups were not significantly different ($P > 0.05$; Fig. 5a, c). On the other hand, the bradycardic response was significantly attenuated by CPF treatment during this regimen period ($P < 0.05$; Fig. 5b). KCN-induced bradycardia at the dose of 40 µg was significantly reduced in CPF-treated animals (CPF 7, $P < 0.01$; CPF 10, $P < 0.05$) when compared with the control group (For details see Online Resource 1).

Considering the 12-week treatment, the chemoreflex hypertensive and tachypneic responses did not differ among treated groups ($P > 0.05$; Fig. 5d, f). Contrasting, KCN-induced reflex bradycardia at the dose of 40 µg (CPF 7, $P < 0.01$; CPF 10, $P < 0.01$) was significantly reduced in CPF-treated animals when compared with the control group (Fig. 5e). At the KCN dose of 80 µg, the bradycardic response of CPF 10 animals was significantly attenuated compared with saline ($P < 0.01$) and CPF 7-treated animals ($P < 0.05$) (Fig. 5e, Online Resource 1).

Basal Respiratory Parameters (V_T, V_E, and f_R)

After 4 weeks of treatment with CPF 10 mg/kg, ($P < 0.01$) basal V_T and basal V_E were significantly increased when compared with the control group (Fig. 6a, b). However, in rats treated for 12 weeks, only V_T was significantly increased by CPF 10 mg/kg ($P < 0.01$), when compared with saline group (Fig. 6d), without significant difference for basal V_E among the 12-week treated groups ($P > 0.05$) (Fig. 6e). Basal f_R did not statistically differ among the groups treated either during 4 weeks ($P > 0.05$) or 12 weeks ($P > 0.05$) (Fig. 6c, f). For statistical details see Online Resource 2.

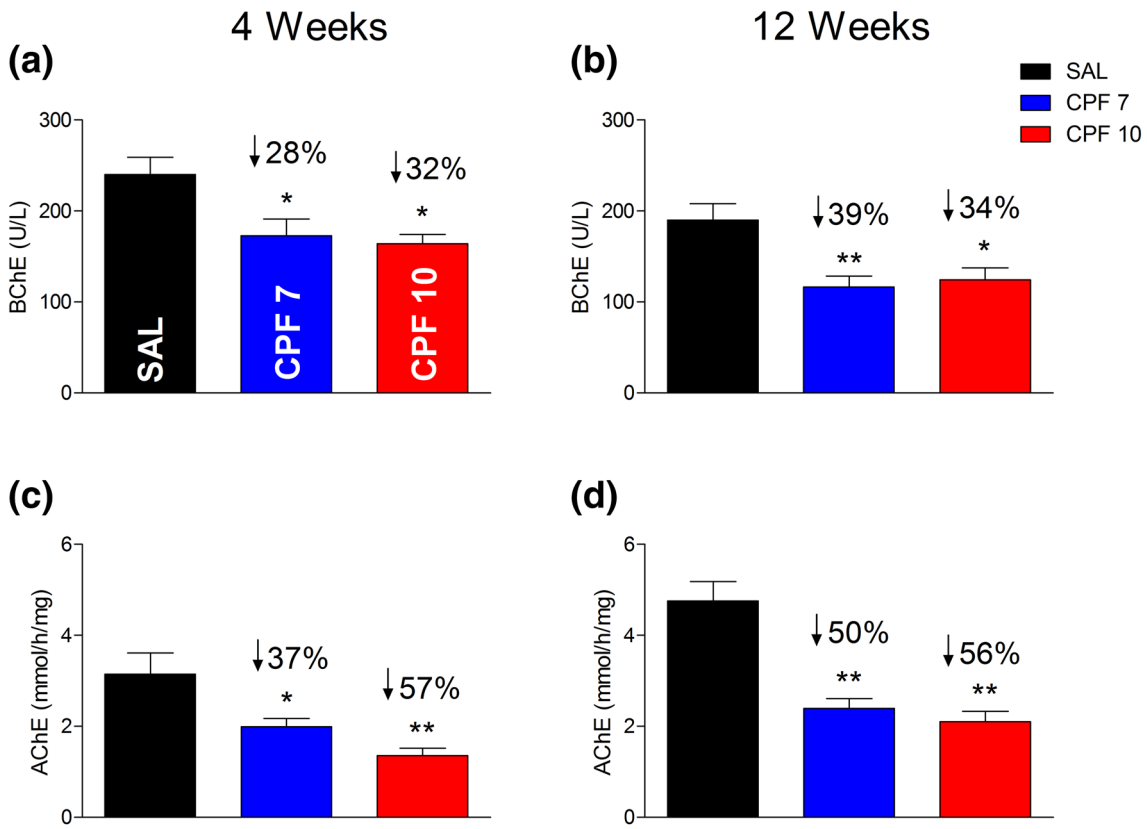

Fig. 2 Enzymatic activity of animals treated with saline (NaCl 0.9%, SAL, black bar) or chlorpyrifos at 7 mg/kg (CPF 7, blue bar) and 10 mg/kg (CPF 10, red bar) during 4 weeks/3 times/week and 12 weeks/once/week. Panel **a** and **b** Plasma butyrylcholinesterase (BChE) activity (U/L) (4 weeks: SAL, $N = 7$; CPF 7, $N = 9$; CPF 10, $N = 7$ and 12 weeks: SAL, $N = 9$; CPF 7, $N = 9$; CPF 10, $N = 9$). Panel **c** and **d** Acetylcholinesterase (AChE) activity in brainstem samples (mmol/h/mg protein) (4 weeks: SAL, $N = 6$; CPF 7, $N = 6$; CPF 10, $N = 6$ and 12 weeks: SAL, $N = 13$; CPF 7, $N = 14$; CPF 10, $N = 12$). One-way ANOVA followed by Bonferroni's post hoc test. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate differences compared with the control group. % reduction in relation to the saline group are presented above the bars

Discussion

We have demonstrated that chronic intermittent exposure to low doses of CPF produced marked changes in the autonomic control of the cardiorespiratory function, characterized by inhibition of the chemoreflex bradycardic responses and of the baroreflex gain, and increase in the VLF band of the HR spectrum, associated with a significant elevation of basal respiratory parameters. These effects coincided with a significant inhibition of plasma BChE and brainstem AChE activity.

Previous studies involving chronic exposure to CPF have employed similar doses to the ones used in the present study [55–58]. Although the present doses were below the maximum tolerated dose, determined in our acute model of intoxication with CPF (30 mg/kg, [26]), our results showed that the 10 mg/kg dose induced some mortality (16.7%) when administered 3 times a week over 4 weeks. On the other hand, no change in weight gain induced by treatment was observed for both periods among CPF-treated and saline groups. Herein, we avoided low doses as described previously in some chronic exposure studies [59] as we wanted to more closely mimic the high levels of exposure experienced by humans working with these products. Additionally, we wished to avoid continuous exposure, as previously performed with CPF [22, 60–63] and with other OP compounds [18, 19, 21, 23–25], but to mimic seasonal exposure by administration of CPF doses intermittently. Intervals between applications as recommended by the vendors for CPF application on crops vary from a few days up to nearly 30 days [11]. Considering that one day of life in the rat corresponds to approximately 30 days of life in humans [64, 65], we chose to expose the animals to CPF with a minimum interval of one day (4 weeks/3 times/week) to cover the reapplication interval usually recommend for crops. Additionally, for the second group we adopted a more widely spaced exposure (weekly for 12 weeks) in order to evaluate whether more disperse intervals of intermittent exposure could differently impact the cardiorespiratory function.

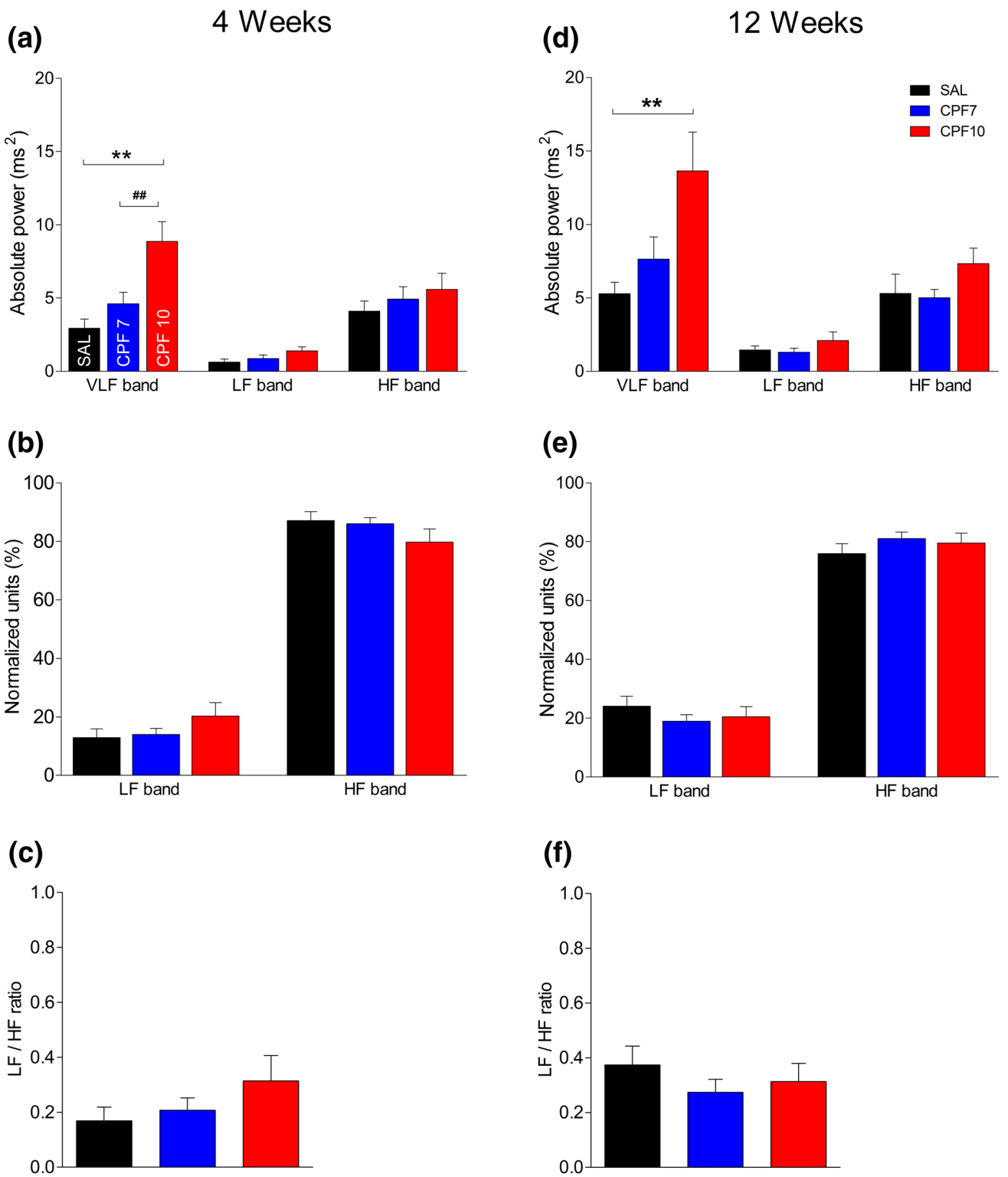

Fig. 3 Heart rate spectral analysis of animals treated with saline (NaCl 0.9%, SAL, black bar) and chlorpyrifos at 7 mg/kg (CPF 7, blue bar) and 10 mg/kg (CPF 10; red bar) of animals treated for 4 weeks/3 times/week (SAL; $N = 9$; CPF 7, $N = 11$; CPF 10, $N = 7$) and 12 weeks/once/week (SAL; $N = 11$; CPF 7, $N = 13$; CPF 10, $N = 10$). Panel **a** and **d** very low-frequency (VLF), low-frequency (LF), and high-frequency (HF) bands in absolute power (ms^2); Panel **b** and **e**: LF and HF bands in normalized units (%); Panel **c** and **f** LF and HF ratio. One-way ANOVA followed by Bonferroni's post hoc test. $^{**}P < 0.01$ indicates differences of CPF and SAL groups and $^{\#\#}P < 0.01$ indicates differences of CPF 7 and CPF 10 groups

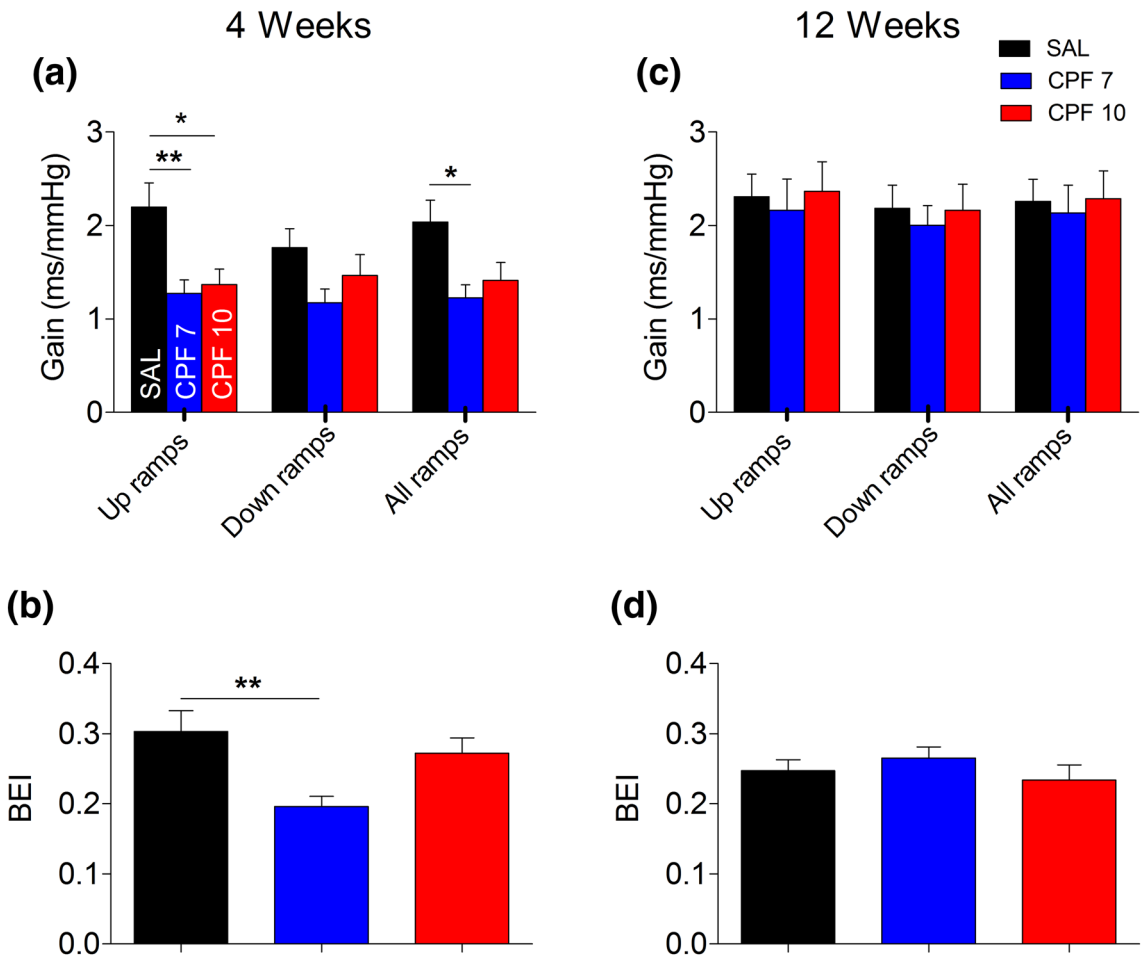

Fig. 4 Spontaneous baroreflex data of animals treated with saline (NaCl 0.9%, SAL, black bar) and chlorpyrifos at 7 mg/kg (CPF 7, blue bar) and 10 mg/kg (CPF 10, red bar) of animals treated for 4 weeks/3 times/week (SAL, $N = 9$; CPF 7, $N = 11$; CPF 10, $N = 7$) and 12 weeks/once/week (SAL, $N = 12$; CPF 7, $N = 12$; CPF 10, $N = 10$). Panel **a** and **c** Baroreflex gain of up ramps, down ramps and all ramps; Panel **b** and **d** Baroreflex effectiveness index (BEI). One-way ANOVA followed by Bonferroni's *post hoc* test. $*P < 0.05$ and $**P < 0.01$ indicate differences of CPF and SAL groups

Although inhibition of plasma BChE does not always correlate with clinical signs of OP intoxication [66], measurement of this enzyme activity has been used as an effective biomarker of OP intoxication in animal models [26, 35, 37, 67]. It also serves as a low-cost screening assay of OP exposure in the clinical setting [68, 69]. Herein, the enzyme activity assays allowed us to confirm that OP exposure had occurred in our rats. Additionally, in studies evaluating cardiac effects induced by OP compounds, the measurement of BChE enzyme is particularly important as reduced levels are associated with mortality in heart disease [70–72].

Our data showed that the CPF treatment significantly reduced BChE activity for all doses and exposure strategies. Although the levels of inhibition obtained were not as marked as observed in CPF acutely poisoned animals [26, 67], it shows that the treatment and doses employed were effective in simulating a scenario of OP exposure. In fact, farmers working in different types of crops either in Brazil or in other countries have experienced similar levels of cholinesterase inhibition during and after the exposure period to OP compounds, including the CPF [73–76], which reinforces that our dose treatment choice in rats is reproducing a phenotype of exposure experience in humans. Moreover, our data show that AChE activity within the brainstem was significantly inhibited for both doses and administration periods, providing further evidence of exposure. In the context of our study, the brainstem was studied as it encompasses most nuclei involved in cardiorespiratory control [77] and the inhibition observed could, therefore, be associated with some of the effects observed on cardiorespiratory function.

Chronic treatment with CPF did not significantly affect basal levels of BP and HR at any dose or exposure regimen. Contrasting data can be found in the literature regarding hemodynamics depending on the compound used, treatment

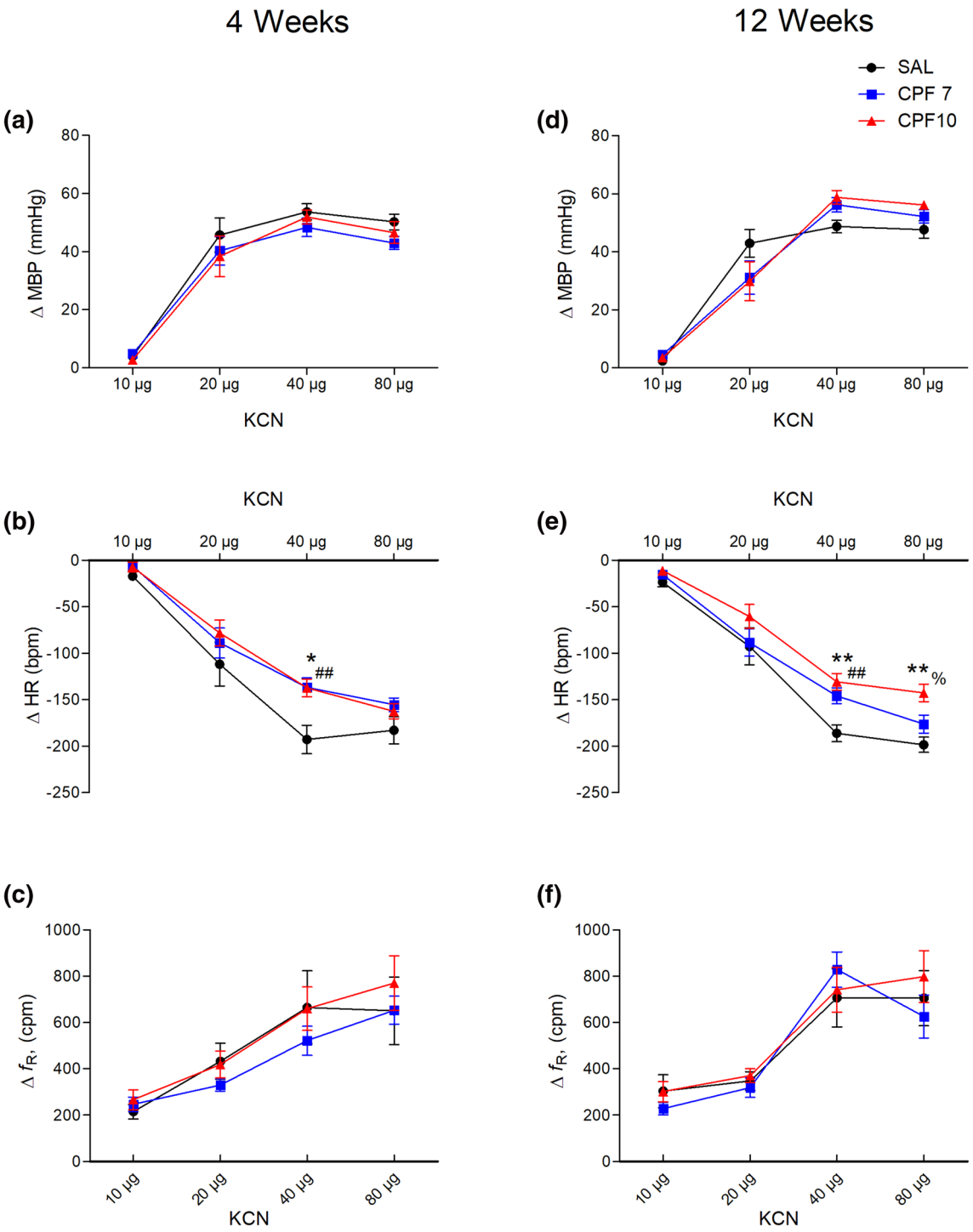

Fig. 5 Changes in mean blood pressure (MBP, Panel **a** and **d**), heart rate (HR, Panel **b** and **e**), and respiratory frequency (f_R, cpm, Panel **c** and **f**) induced by chemoreflex activation with intravenous injections of KCN (10, 20, 40, and 80 µg/rat) in animals treated with saline (NaCl 0.9%, SAL, black circle) or chlorpyrifos at 7 mg/kg (CPF 7, blue square) and 10 mg/kg (CPF 10, red triangle) for 4 weeks/3 times/week (SAL, $N = 9$; CPF 7, $N = 14$; CPF 10, $N = 9$) and 12 weeks/once/week (SAL, $N = 13$; CPF 7, $N = 14$; CPF 10, $N = 12$). Two-way ANOVA followed by Bonferroni's post hoc test. $^{\#\#}P < 0.01$ indicates difference of CPF 7 and SAL groups; $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate differences of CPF 10 and SAL groups; $^{\%}P < 0.05$ indicates difference of CPF 7 and CPF10 groups

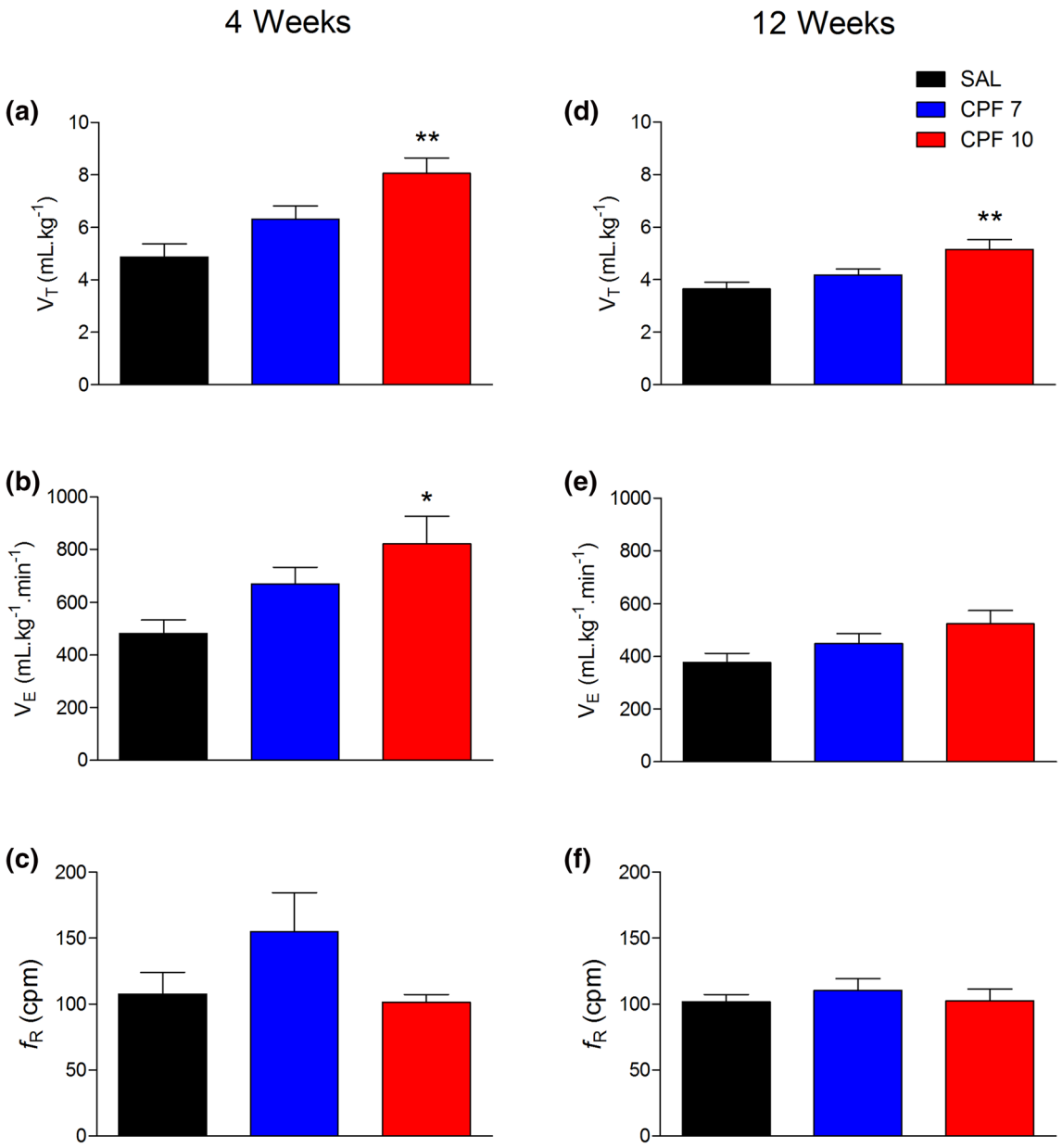

Fig. 6 Basal respiratory parameters of animals treated with saline (NaCl 0.9%, SAL, black bar) or chlorpyrifos at 7 mg/kg (CPF 7, blue bar) and 10 mg/kg (CPF 10, red bar) during 4 weeks/3 times/week (SAL, $N = 9$; CPF 7, $N = 14$; CPF 10, $N = 9$), and 12 weeks/once/week (SAL, $N = 13$; CPF 7, $N = 14$; CPF 10, $N = 12$). Panel **a** and **d** Tidal volume (VT); Panel **b** and **e**: minute ventilation (VE); Panel **c** and **f** basal respiratory frequency (f_R). One-way ANOVA followed by Bonferroni's post hoc test. *$P < 0.05$ and **$P < 0.01$ indicate difference between CPF 10 and SAL groups

period, and doses employed. Maretto et al. [37] did not find any changes in baseline levels in animals acutely exposed to methamidophos (MTP), an OP compound. Similarly, in our previous study [26], acute exposure to CPF at 30 mg/kg in rats did not change basal values of MBP and HR. On the other hand, Smith and Gordon [78] showed an increase in BP, together with tachycardia in rats acutely poisoned with 25 mg/kg CPF. However, in the same study, when animals were acutely injected with a 10 mg/kg dose, as used in our study, no changes in these parameters were observed, despite plasma cholinesterase being significantly inhibited [78]. In the Anthon and Campaña-Salcido [79] study, rats were exposed to three different doses of CPF, via the oral route, for 3 and 14 days and HR did not change, but DBP, SBP, and MBP were increased by the higher doses used. To our knowledge, there are no other studies investigating the effects of repeated and, in particular, intermittent exposure

to OP compounds on cardiovascular and respiratory parameters, preventing further comparison with our data.

Regarding our data on the time domain analysis of SBP and PI variability, there was no change in the variance and standard deviation of these parameters post CPF exposure for both periods of treatment. On the other hand, treatment with CPF increased the VLF band of HR for both periods of treatment, without changing the HF and LF bands. There are not many studies that have investigated the effects of OPs on cardiovascular variability in both the time and frequency domain. Clinical data show that patients who died due to respiratory arrest after acute suicidal poisoning with OPs, including CPF, presented with a marked reduction in the LF and VLF of blood pressure and HR spectrum [80]. However, patients that recovered after intoxication exhibited a marked increase in these spectral bands, suggesting that these parameters may be important prognostic indices to predict mortality post intoxication [80]. In a recent study with patients poisoned with anticholinesterase inhibitors, including OPs and carbamates, the VLF band and the LF/HF ratio were significantly reduced when compared with patients poisoned by other substances, with no differences observed for time domain indices [81]. This data points that exposure to anticholinesterase inhibitors may selectively affect different components assessed in HR variability analysis.

In the experimental field, rats acutely intoxicated with the OP, mevinphos, showed distinct hemodynamic changes, divided in two phases [82]. In the first phase of poisoning, animals showed hypertension and tachycardia, which were accompanied by increases in all components of HR variability: very high-frequency (VHF), HF, LF, and VLF bands. In the later phase, hemodynamic changes turned into a significant hypotension with recovery of VHF and VLF bands to control levels and a reduction of the HF and LF power to levels below baseline. In the same study, direct injection of mevinphos into brainstem regions containing sympathoexcitatory neurons induced the same changes as observed by systemic administration of the OP compound. The authors concluded that the changes observed were due to acetylcholine accumulation induced by the OP that initially resulted in stimulation but reverted to inhibition [82]. Although both clinical and experimental data are quite distinct from our animal model of intermittent repeated exposure, they point out that changes in the HR spectrum depend on the intoxication level and time post exposure and possibly whether continuous versus intermittent, although this needs further validation. Our study demonstrates an important association between changes in HR spectrum and cholinergic transmission within the brainstem.

It is accepted that the HF component of HR spectra reflects parasympathetic modulation, while the LF is influenced by both vagal and sympathetic tone and the LF/HF ratio, therefore, reflects the sympathetic tone [83–87]. There is no consensus for the physiological meaning of the VLF band, as it can be related to circadian and neuroendocrine rhythms, thermoregulation, peripheral vasomotor tone, and hormonal modulation, including the renin–angiotensin–aldosterone (RAA) system [88–90]. It has been shown that atropine almost abolishes this VLF band [90], while pyridostigmine (a reversible cholinesterase inhibitor) strongly potentiates the VLF [91], suggesting that parasympathetic modulation participates on the VLF component. Considering the significant level of AChE inhibition within the brainstem, observed in CPF-treated groups, it is reasonable to speculate that, at least in part, the increase in the VLF band could involve a parasympathetic over-activity or some degree of reduction on sympathetic tone. As above mentioned, VLF band is also strongly modulated by the RAA system [88–90]. Increase in the VLF component induced by CPF could also involve interference in the RAA system once evidence shows that this system is also modulated by long-term exposure to OP [18, 19]. Additionally, there is evidence that slow rhythms (VLF and even ultralow-frequency oscillations) are predictors of worse cardiac outcomes such as cardiac death [92], which reinforces our indications for the toxic effects of intermittent CPF exposure.

CPF injections did not induce changes in the chemoreflex hypertensive response for either 4- or 12-week exposure periods. Bradycardic responses were impaired for both periods and injections employed. Additionally, baroreflex gain was reduced by CPF only after the 4 weeks/3 times/week treatment. Longer intervals between exposures (i.e., 12-week treatment) did not affect this reflex function. Similarly reduction in BEI in CPF-exposed animals was only observed after the 4-week treatment. These data highlight that different intervals between exposure differentially impact the cardiorespiratory function, with a tendency, at least for the baroreflex function, of more disperse intervals between exposure to be less harmful. Additionally, the reduced baroreflex gain associated with impaired BEI also points to an interference in the RAA system and in parasympathetic tone. Evidence shows that the RAA system modulates the baroreflex function and that angiotensin II plays a role in baroreflex impairment in some animal models [93]. Additionally, it has been shown that cardiovascular responses induced by angiotensin system involve increase in cholinergic inputs within the brainstem, suggesting a cross-talk between angiotensinergic and cholinergic pathways [94], which further reinforces the hypothesis of both systems' involvement in the cardiovascular changes observed in the present study.

There are no studies that investigated baro- or chemoreflex function after continuous or intermittent chronic or subchronic exposure to OP compounds. Our previous studies using an acute model of intoxication with CPF [26] and MTP [37] also revealed an impairment of baroreflex- and chemoreflex-evoked bradycardic responses. Regarding an

OP effect on the hypertensive chemoreflex response, this depends on the OP used. While MTP did not affect it in acutely poisoned animals, CPF, at a higher dose (30 mg/kg), reduced the chemoreflex-induced hypertension in acutely poisoned animals [26, 37]. The differences between an intermittent vs acute CPF exposure may rely on different effects on stiffness and vascular tone, as previously described by Guvenc Tuna and colleagues [61].

Considering the chemoreflex tachypneic response, no changes were observed in CPF-exposed animals for both periods of exposure. Similarly, no f_R changes were observed post treatment. In contrast, exposure to the higher concentration of CPF for 4 weeks increased both V_T and V_E, while the 12-week treatment increased only V_T. Thus, longer intervals between exposures also seem to be less harmful to the respiratory function. In cases of acute poisoning by OP compounds, respiratory arrest is one of main causes of death [95]. Houzé and colleagues [96] evaluated the effects of an acute intoxication with the OP—diethylparaoxon, on breathing, and found that this compound increased V_T and reduced f_R in animals. Gaspari and Paydarfar [97] also showed that acute intoxication with dichlorvos reduced f_R and V_T, culminating with apnea in an in vitro model. In other studies, Darwiche and colleagues [98] showed that juvenile and adult rats, chronically exposed to CPF during gestational and post-natal phases, increased the number of sleep apneas, expiratory time, and V_T in the adult phase [98]. Therefore, despite the difference in models, the current evidence shows that OP exposure can affect basal respiratory function either after acute or long-term exposure to these compounds.

The mechanisms involved in the impairment of the reflexes and changes in HR spectrum and in respiratory function remain elusive, but the marked inhibition of AChE activity within the brainstem indicates overstimulation of cholinergic pathways within the main nuclei controlling cardiorespiratory function. Evidence shows that sustained agonist cholinergic stimulation produces desensitization of cholinergic receptors [99–101]. Costa and colleagues [102] also showed that acute or repeated exposure to OPs induces resistance to cholinergic agonist-induced effects. There is also evidence showing that the CPF active metabolite, chlorpyrifos-oxon, binds to M_2 cardiac muscarinic receptors, affecting agonist binding to the receptor [103–105]. Therefore, the impairment observed in the bradycardic reflex responses could, at least in part, involve desensitization or inactivation of cholinergic receptors induced by the OP exposure. Additionally, it is well known that cholinergic circuits within the brainstem play a key role in cardiovascular and respiratory control [95]. It has been shown, for instance, that increases in ACh content within the brainstem, induced by AChE inhibitors, increase respiratory activity in rats [106]. However, it is possible that after repeated exposure (continuous or intermittent) or acute intoxication, the consequent cholinergic overstimulation may lead to failure of neurons involved in cardiorespiratory control [97], which could either contribute to mortality in an acute intoxication or to development of chronic diseases following repeated exposure.

Conclusion

Intermittent exposure to CPF reduced chemoreflex bradycardic responses and baroreflex gain while increased the VLF band of the HR spectrum. This pattern of exposure also induced a significant elevation of basal respiratory parameters. These effects could be associated with cholinergic overstimulation within brainstem circuits regulating cardiorespiratory function as AChE activity was reduced. Additionally, different intervals of intermittent exposure differentially impacted some of the changes observed. This observation raises an important issue when transposing the data to humans emphasizing the need of reducing frequency of exposure and the use of personal protection equipment during exposure to these compounds.

Acknowledgements TJB and ISAF were recipients of CAPES foundation scholarships and AVA was a recipient of a scholarship from the Federal University of Espírito Santo (UFES).

Funding This work was funded by FAPES foundation (Fundação de Amparo à Pesquisa e Inovação do Espírito Santo) (Grant Numbers 67650856/15 and TO131/2019). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)—Finance Code 001.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. All experiments were approved by the Institutional Committee for the Ethical Use of Animals for Research Purposes (CEUA-UFES; Approval Number 36/2016).

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